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The goal of this research project is to explore the role of TGF-B receptors in mammary tumorigenesis and to define the relationship between estrogen receptor and TGF-B receptor signal transduction pathways. We have pursued three approaches in addressing this question: 1) reintroduction of the type II TGF-B receptor into MCF-7 cells resulting in reduced tumorigenicity, 2) characterization of the role p15 and p21, two CdkI's,play in TGF-B mediated signal transduction and 3) the involvement of dwarfins in the signaling cascade downstream of the TGF-B receptors. Further analysis of the inhibitors and dwarfins will not only contribute to our understanding of breast tumorigenesis, but may reveal potential targets for therapeutic intervention.

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Jonathan Wright 9-28-95

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A. INTRODUCTION

One of the most common cancers in woman in the United States is breast cancer. Although endocrine therapy is initially beneficial, the tumors inevitably develop into a hormone insensitive form which no longer responds to the traditional therapies. Therefore, additional markers need to be established to better determine the progress of the lesion. These new markers may lead to improved or alternative therapies. The proposed study of the transforming growth factor-beta (TGF-ß) receptor system and its relationship to the estrogen receptor system may further our understanding of the changes which lead to hormone insensitive growth and present additional targets for therapeutic intervention.

TGF-ß is a multifunctional peptide that plays a role in a wide variety of normal cellular functions including the regulation of proliferation, differentiation, extracellular matrix deposition, cell adhesion and migration (1). However, the effects of TGF-ß can be highly cell-type and even cell-state specific. In normal mammary epithelial cells (NMuMg) TGF-ß acts as apotent cell cycle inhibitor. Loss of TGF-ß's inhibitory actions against cell cycle progression may lead to uncontrollable cellular proliferation (e.g. carcinogenesis). One means by which mammary cells can become TGF-ß insensitive is by down-regulating expression of one or both of the receptors required to mediate the TGF-ß signal. In some systems, the process of receptor down-regulation may be regulated by hormones including estrogen (2,3). MCF-7 cells, an estrogen receptor positive, TGF-ß nonresponsive cell line, can be made TGF-ß responsive by reintroduction of the type II TGF-ß receptor (4). In addition, reestablishment of TGF-ß's potent growth inhibitory pathway reduces the tumorigenicity of MCF-7 cells in nude mice. Some of the downstream molecular events responsible for TGF-ß's growth inhibitory activity are beginning to be determined (1). We have initiated a study aimed at determining if these same events are occurring in MCF-7 cells when TGF-ß's growth inhibitory pathway is restored.

Elucidation of the pathway(s) which mediate TGF-ß's growth inhibitory signal may reveal promising targets for therapeutic intervention. In an attempt to identify other molecules within TGF-ß's signal transduction pathway we have initiated a collaboration with Dr. Richard Padgett at Rutger's University. Dr. Padgett uses *C. elegans* to genetically determine components of the dauer larva formation pathway. The receptors that initiate this signal in *C. elegans* are TGF-ß receptor homologs. Thus *C. elegans* serves as a useful genetic tool to identify potential downstream effectors for TGF-ß in mammalian cells.

In the past twelve months our focus has been on the reestablishment of TGF-ß responsiveness in MCF-7 cells, development of a system to determine the interrelationships of the estrogen pathway and the TGF-ß pathway and investigation of the role dwarfins play in TGF-ß's signal transduction pathway.

B. PROGRESS REPORT

Many breat cancer cell lines have lost the ability to respond to TGF-ß's growth inhibitory signal. Generally, the type II receptor has been found to be expressed at very low or even undetectable levels in these cells. Therefore, in collaboration with Dr. M. Brittain's lab at the Medical College of Ohio, we have examined the effect of reestablishing the TGF-ß pathway in a nonresponsiveness cell line, MCF-7. The parental MCF-7 cells were shown to lack detectable levels of the type II TGF-ß receptor (RII). Stable transfection of the RII cDNA in a mammalian expression vector yielded three clones with varying levels of exogenous RII expression. ^{125}I-TGF-ß labelling of the transfected cells also showed an increase in binding to the type I TGF-ß receptor (RI). Therefore, only the type II receptor is absent in the MCF-7 cells, but in the absence of RII, RI is incapable of binding TGF-ß. The MCF-7 RII transfectants were growth inhibited in a dose-dependent manner by TGF-ß, but the control clones remained TGF-ß resistant. To determine the effect of RII expression on the tumorigenicity of MCF-7 cells saturation density, clonogenic growth in soft agar and tumorigenicity in ovariectomized estrogen-supplemented nude mice were examined. The RII transfectants growth arrested at saturation densities that were 41-66% less than control cells when grown in monolayer culture. Soft agar clonogenicity was reduced and tumorigenicity in nude mice was reduced and delayed in correlation with the amount of RII expression in each of the clones; higher RII expression led to a greater reduction in tumor formation and a longer lag before tumor growth. In fact, examination of the tumors that developed from RII transfected MCF-7 cells revealed that these tumors had lost exogenous RII expression. These studies indicate that reexpression of the type II TGF-ß receptor in transformed cells that retain TGF-ß's signal transduction components can reverse the malignant phenotype of breast cancer cells (4).

The importance of TGF-ß in maintaining the delicate balance required for normal cellular proliferation has also recently been demonstrated in hereditary nonpolyposis colorectal cancers (HNPCC). The RER colorectal cancer genotype which is characteristic of HNPCC, is associated with TGF-ß insensitivity due to a loss of type II TGF-ß receptor expression. In collaboration with Dr. Brittain's lab, RII was reintroduced into RER colon carcinoma cells with similar results to the MCF-7 study. The transfectants showed reduced saturation densities, reduced clonogenicity in soft agar and reduced and delayed tumorigenicity in nude mice. These two studies highlight the critical role that the type II TGF-ß receptor plays in tumorigenesis of breast cancer and HNPCC (5,6).

The signal transducing molecules downstream of the TGF-ß receptors have yet to be identified. However, some of the nuclear effectors of TGF-ß's growth inhibitory signal have been determined. These effectors belong to a growing family of proteins termed the cyclin-dependent kinase inhibitors, CdkI's. Progression through the cell cycle requires an orderly activation and subsequent inactivation of a variety of cyclin/cdk complexes. The CdkI's bind to either the cyclin/cdk complex or block association of the cyclin with its Cdk partner, thus inhibiting cyclin/Cdk activity. Three CdkI's have been shown to be involved in TGF-ß's cell cycle arrest at the G1/S boundary. These are p27, p21 and p15. The best characterized of these inhibitors is p21.

Three independent methods were used to clone p21: 1) the two-hybrid system using cdk2 as bait; 2) subtractive hybridization for molecules induced by p53; and 3) by microsequencing a 21 kDa protein present in cyclin/cdk complex immunoprecipitations. p21 was subsequently shown to inhibit all cyclin/cdk complexes, to be induced by p53 and to cause a G1 phase cell cycle arrest when overexpressed. All three of these characteristics of p21 made it an excellent candidate for an effector of TGF-ß's growth inhibitory signal. Initial studies in TGF-ß responsive human keratinocytes showed that p21 mRNA and protein levels were increased following TGF-ß treatment. It was further

demonstrated that TGF-ß induces transcription of p21 which leads to the increase in p21 protein levels (7). Transcriptional induction of another CdkI, p15, has also been observed following TGF-ß treatment of HaCat cells (8). These results indicate that a variety of TGF-ß effector molecules are induced to inhibit the cyclin/cdk activities which are required for cell cycle progression ultimately resulting in TGF-ß's G1 phase cell cycle arrest.

The demonstration that CdkI's (p15, p21 and p27) play a role in TGF-ß induced cell cycle arrest, led us to ask if a similar mechanism was involved in TGF-ß mediated growth inhibition of mammary cells. We are currently using two cell lines as model systems to examine the role of CdkI's in TGF-ß's growth inhibitory pathway, normal mammary epithelial cells (NMuMg, CRL1636) and the MCF-7 RII transfectants described earlier. Our preliminary Western results show that the MCF-7 RII transfectants up-regulate p21 protein levels. This is supported by transient transfection assays which show the p21 promoter-luciferase reporter construct is induced following TGF-ß treatment. These studies are being extended by examining the levels of p15 and the activity of p27 in both systems following TGF-ß treatment. In addition, the effect of estrogen treatment on the ability of TGF-ß to cause a G1 phase arrest in these systems will be determined. The ability or inability of TGF-ß to generate its growth inhibitory signal in the presence of estrogen will be correlated with the effects on induction and activity of the CdkI's. This approach will allow for initial dissection of the relationship between the TGF-ß receptor pathway and the estrogen receptor pathway.

Between the nucleus, where CdkI's play a role in TGF-ß's growth inhibitory signal, and the membrane, where TGF-ß binds and activates its receptors, there exists an unknown signaling cascade which is responsible for transfering TGF-ß's message to stop the cell cycle. Elucidation of the pathway is certain to reveal additional targets for therapeutic intervention by virtue of their involvement in TGF-ß's signaling. The process of deciphering the steps along the pathway from the membrane to the nucleus is extremely difficult in mammalian systems. However, the use of a genetically tractable organism such as *C. elegans* can readily provide potential candidates for the pathway. Three such genes have recently been identified to play a role in daf-4 signaling, *sma-2*, *sma-3* and *sma-4*. Interestingly, the three genes are greater than 90% identical and yet are incapable of functionally rescuing one another in *C. elegans*. This suggests that all three are somehow required to transduce daf-4's signal. Homologs for these genes are detectable in murine and human cDNA libraries and have been renamed dwarfins (Dwfs). At present only the cDNA for murine Dwf-A has been isolated in near-full length form; the clone lacks a few amino acids at the amino terminus, but contains the two highly conserved dwarfin homology domains, DH1 and DH2. Preliminary results indicate that the phosphorylation state of a transfected tagged-Dwf-A molecule changes upon TGF-ß treatment in COS and Mv1Lu cells. This result would suggest an involvement of Dwf-A in TGF-ß's signal transduction pathway.

If this result is confirmed in COS and Mv1Lu cells, the breast cell lines will be examined for potential involvement of Dwf-A in TGF-ß mediated signals. Dwf-A has already been shown to be expressed in the NMuMg cell line by an RNase protection assay. A time course of TGF-ß treatment from 1 hour to 24 hours shows no alteration in the mRNA levels for Dwf-A. Therefore, involvement in the pathway would require a change in state or activity for the Dwf-A molecule like that observed in COS and Mv1Lu cells. Our effort with regard to the dwarfins will consist of several approaches aimed at determining if indeed they are involved in TGF-ß's signaling cascade: 1) to clone the full length form of all three dwarfins (Dwf-A, Dwf-B and Dwf-C), 2) determine their tissue distribution in mammalian organisms, 3) determine the effect of overexpressing each Dwf individually and in combination on TGF-ß-dependent reporter gene activation and growth inhibition and 4) expression of dominant negative forms of the Dwfs to antagonize TGF-ß activity. If the dwarfins are shown to be involved in the TGF-ß pathway, they would be the first cytoplasmic TGF-ß signaling molecules to be identified and would provide not

only potential targets for therapeutic intervention in breast cancer, but valuable tools to identify other molecules involved in TGF-ß's signaling pathway (e.g. the kinase which phosphorylates Dwf-A).

C. CONCLUSIONS

The discovery that loss of the type II TGF-ß receptor in breast cancer cell lines and RER colorectal carcinomas contributes to tumorigenesis underscores the crucial role TGF-ß plays in maintaining normal progression through the cell cycle. Reduced expression or loss of this one key check on the cell cycle contributes to the multistep process of carcinogenesis. The model system developed can be used to further characterize the molecular basis for TGF-ß's growth inhibitory signal and to determine the relationship between the estrogen pathway and the TGF-ß pathway in both normal (NMuMg) and malignant cells (MCF-7).

Identification of several nuclear effectors of TGF-ß, the CdkI's p15, p21 and p27, enables a more detailed analysis of the mechanisms involved in TGF-ß's effect on breast epithelial cells. They also provide useful markers for determining at what level estrogen may be interfering with TGF-ß's ability to stop the cell cycle. Careful examination of the role of each CdkI in breast epithelial cells will not only contribute to our understanding of TGF-ß signaling mechanisms, but more importantly further our understanding of the mechanisms involved in breast tumorigenesis.

Finally, determination of the role dwarfins play in TGF-ß signal transduction in general and more specifically in breast epithelia will provide additional targets for therapeutic strategies aimed at controlling the cell cycle of transformed cells. By identifying a cytoplasmic intermediate in the TGF-ß pathway the opportunity will exist to work up towards the receptors and down towards the nucleus simultaneously in hopes of connecing TGF-ß's signal at the membrane with its ultimate effects in the nucleus, cell cycle control and gene induction.

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